THE
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JOURNAL

Lipid induced lysosomal damage after demyelination corrupts microglia protective function in Lysosomal Storage Disorders

Enrique Gabandé-Rodríguez, Azucena Pérez-Cañamás, Beatriz Soto-Huelin, Daniel N. Mitroi, Sara Sánchez-Redondo, Elena Martínez-Sáez, César Venero, Héctor Peinado and María Dolores Ledesma.

Editor: Elisabetta Argenzio

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision   17th May 2018

Thank you for submitting your manuscript (EMBOJ-2018-99553) on the protective roles of microglia in lysosomal storage disorders to The EMBO Journal. We have now received three referee reports on your study, which are enclosed below for your information.

As you can see, while the referees judge the findings to be overall important and interesting, they also raise critical points that need to be addressed before they can support publication at The EMBO Journal. In particular, referee #1 is concerned that the study suffers from misinterpretation of your results on neurotoxicity of CathepsinB release and suggests expanding the characterization of pro- versus anti-inflammatory microglia. In addition, referee #3 points to concerns regarding causalities between demyelination and microglia, as well as the physiological relevance of the findings obtained with BMDM cells.

Addressing these issues as suggested by the referees would be essential to warrant publication in The EMBO Journal. Given the overall interest of your study, I would thus like to invite you to revise the manuscript in response to the referee reports.

REFEREE REPORTS

Referee #1:

The authors study the contribution of microglia in the pathogenesis of the lysosomal storage disorder caused by deficiency of acid sphingomyelinase (ASM) in mice. ASM KO mice are characterized by an increase in microglia number an activation status. Ablation of microglia using

the compound PLX5622 worsen the disease as measured by behavioral, lethality and morphological analyses. Using markers against Arginase1 and CD16 or F4/80, the authors find activated microglia polarized towards both "M1" and "M2" phenotypes. The Arginase1 positive microglia are the cells that primarily engulf myelin debris. A fraction of these cells exhibited signs of lysosomal damage as shown by CathepsinB release. Medium transfer experiments show that microglia releasing CathepsinB exert neurotoxic effects, which was rescued by a CathepsinB inhibitor both in vitro and in vivo. Finally, the authors extend their findings to NPC, a second lysosomal storage disease. In summary, this paper enhances our understanding of lysosomal storage diseases and provides a novel therapeutic strategy. The experiments are convincing and of high quality. Overall, the paper is of great interest and I therefore recommend publication after considering the following points:

My main criticisms concern the interpretation of some of the results. I am not fully convinced that the release of CathepsinB from microglia is directly neurotoxic. There is evidence that lysosomal damage leads to release of CathepsinB into the cytosol of phagocytes, where it can activate the inflammasome pathway (Duewell et al. Nature 2010), which in turn could mediate the neurotoxic effects for example via Il1b. The authors should discuss this possibility in the discussion.

The authors classify the microglia into pro- and anti-inflammatory cells based on single markers. If the authors want to keep this nomenclature more work is necessary. The authors would need to use additional markers and in particular markers that indicate pro- and anti-inflammatory changes. Alternatively, the cells could be isolated an analyzed by RNA-Seq. However, as these pro- and anti-inflammatory pathways are not part of this study, I recommend avoiding these labels.

The authors refer to sphingomyelin as a lipid enriched in myelin. In spite of its name, sphingomyelin is depleted from myelin.

Page9: "We found diffuse CathB staining in a significant percentage of Iba1 positive cells in the Cb from ASMko but not wt mice, which supports LMP in ASMko microglia (Figure 4 F, H)". Additional assays are necessary to prove this point. Increased labeling is easy to see, but the diffuse labeling is not sufficient to prove lysosomal membrane rupture. The authors could analyze lysosomal pH or determine biochemically the cytosolic fraction of CathepsinB or use other assays. The authors should in addition, test whether microglia become TUNEL positive. Release of Cathepsin into the cytosol is known to trigger pyroptosis.

The authors ablate microglia and show that this leads to worsening of the disease. Ablation of cells leads to release of potentially cytotoxic cell debris. This possibility should be discussed as a reason for the deterioration of the disease.

Referee #2:

In this article the authors studied the role of microglia in the evolution of neuropathic Lysosomal storage disorders (LSDs) as Nieman Pick disease. in order to elucidate the role of microglia in the development of this disease the authors studied patient samples and a sphingomyelinase knockout mouse model (ASMko). By studying the morphology of the cells among different brain regions the authors showed that microglia acquires an amoeboid shape due to their activation. The most unexpected results is that the depletion of microglia increase the pathological symptoms in the KO mouse model, suggesting a neuroprotective contribution of the microglia. However the authors were able to show the existence of two microglia phenotypes based on Cathepsin B (CathB) and Lamp1 expression. Revealing the presence of microglia phagocyting large amount of myelin debris inducing an overloadding of the lyzosomes and their exocytosis to the extracellular space, the main consequence being the release of CathB playing a neurotoxic role in the evolution of the disease. The authors succeed to show that inhibiting CathB activity in this mouse model reduces neuronal death and behavioral anomalies.

The article is well written and clear. All the informations necessary to understand and follow are cited. The experiments are well design and controls are there. All the results are presented in a very clear manner and not over interpreted.

I have only few comments:

- In the introduction the author should refer to the uniqueness of microglia origin (Ginhoux et al 2010).
- Following on this, how the authors can formally exclude that the cells that they are looking are monocyte-derived cells that colonize the brain and not embryonic derived microglia that could die

due to the KO.

- In the discussion the authors could discuss about the recent description of the disease-associated microglia, also called DAM, performed by Keren-Shaul et al 2017 and how this could be related to the microglia they described in this article.

Referee #3:

EMBOJ-2018-99553, corr. author Dr. Ledesma
"Lipid induced lysosomal damage after demyelination corrupts microglia protective function in Lysosomal Storage Disorders"

Reviewer comment:
The manuscript 'Lipid induced lysosomal damage after demyelination corrupts microglia protective function in Lysosomal Storage Disorder' nicely demonstrates both the protective as well as the detrimental effect of microglia in Niemann Pick disease. Although it was already known that microglia phagocytose myelin debris thereby promoting remyelination, Gabandé-Rodríguez et al. show here for the first time with different in vitro and in vivo models and in a human patient that microglial Cathepsin B release from the lysosomes is contributing to neuronal degradation in NPA and NPC. Furthermore, they demonstrate that inhibition of Cathepsin B release ameliorates disease symptoms like Purkinje cell death and motor deficiencies.
The manuscript is well structured and written and the topic - dissecting the effect of pro- and anti-inflammatory microglia populations and their functions in LSD - is certainly of high interest. However, there are a couple of major concerns, which should be addressed:

Major points.
1. Supplementary Figure S1: To exclusively link the demyelination process to the microglia, one would need to not only count the number of oligodendrocytes, but rather assess their myelination activity, i.e. by analyzing mRNA/protein levels of MBP, PLP or DM20 in isolated oligodendrocytes.
2. Figure 4E; Fig. 5; Supplementary Figure S4, : The authors state that "We used BMDMs as they share many characteristics with microglia, but they can be cultured from adult ASMko mice when SM accumulation is evident unlike microglia (Ledesma et al, 2011)"
This explanation regarding the use of BMDMs is not satisfying. Adult microglia from different mouse models can be isolated and cultured for at least 24 hours (see e.g. Trias et al., 2013 Front Cell Neurosci. 2013 Dec 24;7:274; Wagner et al., 2017, Acta Neuropathol Commun. 2017 Jun 24;5(1):52 or Bornstein et al., 2013 J Vis Exp. 2013; (78): 50647). Furthermore, the cited reference does not discuss macrophages or microglia of ASMko mice. Thus, confirmation of the BMDM data by using adult microglia (and perhaps adult astrocytes or oligodendrocytes as negative control) would increase the impact of these findings.

Minor points.
1. We suggest to change 'lysosomal storage disorders' in the title into 'Niemann Pick disease', since this is what the data can corroborate.
2. Fig 1A: please provide a magnified image for visualization of microglial morphology.
3. Fig 2A, the staining seems very weak and with a high background. Also please provide a magnified image.
4. Fig 3E, only shows two groups although there are 6 groups in the legend.
5. Fig 4F,G: please provide a magnified image.
6. Fig 5C,J: please provide a magnified image.
7. Fig. 5E, please show the entire Ponceau S blot.
8. Fig 5G&H, please provide a housekeeping protein/Ponceau S for these blots as well.
9. Fig. 5J, an overlay of the two channels will help to appreciate the co-localization.
10. Fig 7H,I: please provide a magnified image.
11. Fig. 7a, why are there no Iba1 positive cells visible in the wt cerebellum?
12. Fig. S5, why is there no double stain of the NPA patient for Iba1 and CathB, like there is for the

NPC patient? It is impossible to tell from this chromogenic stain whether the CathB shown is found in microglia or neurons.

13. Description of osmotic mini-pump on page 11: it appears from the sentence that the Ca074Me was released s.c., shouldn't that be i.c.v?

1st Revision - authors' response   7th September 2018

EMBOJ-2018-99553
POINT-BY-POINT ANSWER TO REVIEWERS

Referee #1:
We thank this reviewer for considering our study of great interest and high quality.

I am not fully convinced that the release of CathepsinB from microglia is directly neurotoxic. There is evidence that lysosomal damage leads to release of CathepsinB into the cytosol of phagocytes, where it can activate the inflammasome pathway (Duewell et al. Nature 2010), which in turn could mediate the neurotoxic effects for example via Il1b. The authors should discuss this possibility in the discussion.

The reviewer is right Cathepsin B can cause toxicity directly or indirectly through the activation of the inflammasome pathway. However, we would like to stress that in our toxicity experiments we added the non-membrane permeable CathepsinB inhibitor Ca074 to the media after collecting it from the macrophage cultures, which were never directly exposed to the inhibitor. Therefore, our experimental setting does not allow counteracting the inflammasome response of the macrophages and targets specifically the CathepsinB that has been already secreted. Nonetheless, and to better address the interesting point raised by this reviewer, we have measured the level of IL1b in the brains of ASMko and wt mice. The finding that the level of this main component of the inflammasome response is reduced, and not increased, in the ASMko mice would argue against the activation of the pathway in the ASMko conditions. As requested by the reviewer this is now discussed in the highlighted text in page 17 and the results shown in the new Supplementary Figure S10.

The authors classify the microglia into pro- and anti-inflammatory cells based on single markers. If the authors want to keep this nomenclature more work is necessary. The authors would need to use additional markers and in particular markers that indicate pro- and anti-inflammatory changes. Alternatively, the cells could be isolated an analyzed by RNA-Seq. However, as these pro- and anti-inflammatory pathways are not part of this study, I recommend avoiding these labels.

We agree with the reviewer that the detailed pro and anti-inflammatory pathways are not the focus of this study. Following his/her recommendation we have avoided these labels throughout the text referring now to different microglia phenotypes/populations and to Arg-1 or CD16/CD32 positive microglia instead of pro and anti-inflammatory microglia.

The authors refer to sphingomyelin as a lipid enriched in myelin. In spite of its name, sphingomyelin is depleted from myelin.
It is true that, although having a key role in myelin integrity and stability, sphingomyelin is a relatively minor myelin component compared to cholesterol or cerebrosides. We apologize for this misleading sentence in which we have deleted the term "enriched" to address this reviewer comment.

Page9: "We found diffuse CathB staining in a significant percentage of Iba1 positive cells in the Cb from ASMko but not wt mice, which supports LMP in ASMko microglia (Figure 4 F, H)". Additional assays are necessary to prove this point. Increased labeling is easy to see, but the diffuse labeling is not sufficient to prove lysosomal

membrane rupture. The authors could analyze lysosomal pH or determine biochemically the cytosolic fraction of CathepsinB or use other assays.

To address this reviewer query we have analysed CathepsinB levels in the cellular and cytosolic fractions of ASMko and wt BMDMs extracted with digitonin. Consistent with LMP we found increased CathB in the cytosol of ASMko BMDMs. Moreover, we have cultured microglia derived from wt and ASMko mice and analysed lysosomal integrity by lysotracker staining finding a diffuse pattern also consistent with LMP. These results are described in the highlighted text in pages 9-10 and shown in the new Figure 4F and the new Supplementary Figure S5.

The authors should in addition, test whether microglia become TUNEL positive.

To analyse microglia death we have used both cleaved Caspase-3 staining and TUNEL assays in combination with microglia identification by F4/80 labelling in the cerebellum of ASMko and wt mice. While we find increased apoptosis in the total cell population of the ASMko mice, we do not detect a significant increase neither in the number of Cleaved-Caspase-3 nor in TUNEL positive F4/80 cells. This suggests that microglia do not undergo apoptosis in the disease. We show these results in the new Supplementary Figure S9 and discuss them in the highlighted text in page 17.

Release of Cathepsin into the cytosol is known to trigger pyroptosis. The authors ablate microglia and show that this leads to worsening of the disease. Ablation of cells leads to release of potentially cytotoxic cell debris. This possibility should be discussed as a reason for the deterioration of the disease.

We thank this reviewer for this interesting comment as indeed pyroptosis and cytotoxic cell debris might worsen the disease. While we cannot completely rule out these possibilities several observations would argue against them. On one hand, we have analysed the levels of IL-1b, whose release is directly linked with pyroptosis, finding a reduction and not an increase in the brain of ASMko mice (new Supplementary Figure S10). On the other hand, it has been described that ablation of microglia by the CSF1R inhibitor PLX occurs by apoptosis very rapidly (3-5 days) after treatment initiation and have no toxic effects in wt mice. We confirmed the lack of toxicity in the wt mice after 2-month long PLX treatment. This makes unlikely that cell debris released from microglia ablation, which would appear in both wt and ASMko mice at very early stages of the treatment, is deleterious only in the ASMko mice. We discuss these issues in the highlighted text in pages 14-15 and 17.

Referee #2:
We thank this reviewer for his/her kind words on the clarity and interpretation of our results.

In the introduction the author should refer to the uniqueness of microglia origin (Ginhoux et al 2010).
We now refer to the uniqueness of microglia origin in the highlighted text in page 4.

Following on this, how the authors can formally exclude that the cells that they are looking are monocyte-derived cells that colonize the brain and not embryonic derived microglia that could die due to the KO.
The reviewer is right we cannot exclude the presence of monocyte-derived cells in the brain of ASMko mice. To address this issue we have performed staining against the transmembrane receptor TMEM119, which preferentially labels microglia and not

macrophages. We indeed see that a percentage of the F4/80 positive cells do not express TMEM119 or express it at low levels supporting their macrophage identity (but see answer to the next point on DAM microglia). The data we obtained in cultured ASMko BMDMs and the new analysis performed in primary cultured ASMko microglia confirm that both cell types show lysosomal membrane permeabilisation and Cathepsin B release. Therefore, they would similarly contribute to neuronal death irrespective of their origin. These results are shown and discussed in the highlighted text in pages 10 and 15 and in the new Supplementary Figure S6.

In the discussion the authors could discuss about the recent description of the disease- associated microglia, also called DAM, performed by Keren-Shaul et al 2017 and how this could be related to the microglia they described in this article.

We thank this reviewer for this interesting remark. It could certainly be that the protective disease-associated microglia (DAM), which have been recently described in mouse models for Alzheimer´s disease and amyotrophic lateral sclerosis, also play a role in Niemann Pick diseases. Even more so as these cells upregulate Cathepsin B. We have tried to identify this microglia population in the ASMko mouse brain using antibodies against the DAM-enriched protein Trem2. However, the slight differences in intensity we have found do not allow establishing solid conclusions on the presence of DAM microglia that would require transcriptional single cell sorting. Interestingly, the expression of the microglia marker TMEM119 is downregulated in DAM microglia. It might be that the F4/80 positive cells showing none or low TMEM119 expression in the ASMko mouse brains are not infiltrated macrophages (see above point for this reviewer) but DAM microglia. This would be in agreement with very recent reports describing the absence of infiltrating peripheral myeloid cells in the brains of different mouse models for LSDs including NPC (Cho et al., 2018; Cougnoux et al., 2018). Addressing the reviewer query we now discuss these issues in pages 10 and 15 and show the results in the new Supplementary Figure S6.

Referee #3:
We thank this reviewer for acknowledging the high interest of the topic of our study.

Major points. 1. Supplementary Figure S1: To exclusively link the demyelination process to the microglia, one would need to not only count the number of oligodendrocytes, but rather assess their myelination activity, i.e. by analyzing mRNA/protein levels of MBP, PLP or DM20 in isolated oligodendrocytes.

We do not intend to exclusively link the demyelination process to the microglia. In fact, we propose that demyelination is the cause, and not the consequence, of microglia alterations since myelin debris overwhelm the phagocytic capacity of these cells damaging their lysosomes and promoting the subsequent release of toxic Cathepsin B. While it is not the scope of our study we agree with this reviewer on the relevance of understanding the reason why demyelination occurs. Buccina et al., (J Neurochemistry 2009) already analysed mRNA and expression levels of the myelin-specific proteins MBP, MAG, CNP and PLP –DM20 at birth and at early postnatal stages in ASMko mice. They found reduced mRNA and protein levels for all of them at 4 and 10 weeks of age but not at birth, when transcription factors involved in oligodendrocyte (OL) development were also found unaltered. These results favoured the hypothesis of altered myelin maintenance rather than deficient OL generation or development in ASMko conditions. This needs further investigation but to address this reviewer query we have

assessed the myelination capacity of OLs by co-staining of MBP and the axonal marker Neurofilament 200 in wt and ASMko mice treated or not with the microglia ablating drug PLX5622. The number of axons not wrapped by myelin in ASMko mice is increased compared to wt. This feature is not affected by PLX5622 treatment. This suggests that the myelination capacity of ASMko OLs is impaired but not influenced by microglia. These results are now shown and discussed in the new Supplementary Figure S2 and in the highlighted text in page 8.

2. Figure 4E; Fig. 5; Supplementary Figure S4, : The authors state that "We used BMDMs as they share many characteristics with microglia, but they can be cultured from adult ASMko mice when SM accumulation is evident unlike microglia (Ledesma et al, 2011)" This explanation regarding the use of BMDMs is not satisfying. Adult microglia from different mouse models can be isolated and cultured for at least 24 hours (see e.g. Trias et al., 2013 Front Cell Neurosci. 2013 Dec 24;7:274; Wagner et al., 2017, Acta Neuropathol Commun. 2017 Jun 24;5(1):52 or Bornstein et al., 2013 J Vis Exp. 2013; (78): 50647). Furthermore, the cited reference does not discuss macrophages or microglia of ASMko mice. Thus, confirmation of the BMDM data by using adult microglia (and perhaps adult astrocytes or oligodendrocytes as negative control) would increase the impact of these findings.

We apologize for the mistake in the reference citation that we have now corrected. Following this reviewer suggestion we have tried hard to culture adult microglia from ASMko mouse whole brain.

Unfortunately, and in agreement with the previous reports, the cellular yield was quite low. Due to the high number of cells required to detect Cathepsin B in the media we have not been able to obtain results from those cultures. In order to satisfy this reviewer query we have set up two alternative experimental approaches; a) post-natal derived microglia cultures from wt and ASMko mice in which we have confirmed the accumulation of SM after 14 days in culture by Lysenin staining. Similar to the ASMko BMDMs, these cells showed diffuse cytosolic staining of Lysotracker and the presence of extracellular cathepsin B in the culture media; b) organotypic cerebellar slice cultures from adult ASMko mice fed with PLX5622 to ablate microglia or with vehicle diet. We have analysed the presence of Cathepsin B in the media of the slice cultures finding an increase in the vehicle treated but not in the PLX treated ASMko mice. These results strongly support adult ASMko microglia as a source of secreted Cathepsin B and are described in the highlighted text in page 12 and in the new Figures 5N,O,P and in the new Supplementary Figure S5.

Minor points.
1. We suggest to change 'lysosomal storage disorders' in the title into 'Niemann Pick disease', since this is what the data can corroborate.
We will be willing to change the title if the referee considers it necessary. However, we would like to remark that, although with similar names, Niemann Pick diseases type A and C are two completely different LSDs caused by different genetic defects. Still, we find a similar phenotype induced by demyelinaton and lysosomal lipid accumulation, which are characteristics shared by many other LSDs. This moves us to propose that this is a general pathological mechanism in LSDs and to kindly ask this referee to allow us reflecting this in the title.

2. Fig 1A: please provide a magnified image for visualization of microglial morphology.

A magnified image of microglia morphology is now included

3. Fig 2A, the staining seems very weak and with a high background. Also please provide a magnified image.
We have improved the staining and provide a magnified image

4. Fig 3E, only shows two groups although there are 6 groups in the legend.

The graph in Fig 3E included data from all 6 groups but the merge of the lines did not allow distinguishing them. We have modified the graph to clearly show all groups.

5. Fig 4F,G: please provide a magnified image.

We provide a magnified image

6. Fig 5C,J: please provide a magnified image.

We provide a magnified image in Fig 5J. In Figure 5C there was already a magnified image. We apologize for not having reflected this in the legend. We have now corrected the text to make this point clear

7. Fig. 5E, please show the entire Ponceau S blot.

We show the entire Ponceau S blot in the new Supplementary figure S11

8. Fig 5G&H, please provide a housekeeping protein/Ponceau S for these blots as well.

The Western blots in Figures 5G and H correspond to the secreted exosome fraction and the exosome depleted culture media, respectively, from wt and ASMko BMDMs. These fractions contained two little protein to be detected by Ponceau S and we are not aware of a housekeeping protein that we could use as loading control for the culture media. However, we would like to note that the experimental setting we use provides with internal controls for protein amount since we

cultured the same number of cells in all conditions and since the exosome enriched and depleted fractions come from the very same culture media sample in each case.

9. Fig. 5J, an overlay of the two channels will help to appreciate the co-localization.

We now provide the overlay of the two channels.

10. Fig 7H,I: please provide a magnified image.

We provide a magnified image.

11. Fig. 7a, why are there no Iba1 positive cells visible in the wt cerebellum?

We apologize for the poor quality of the original image. We now provide a new one that better reflects the iba1 positive cells in the wt cerebellum.

12. Fig. S5, why is there no double stain of the NPA patient for Iba1 and CathB, like there is for the NPC patient? It is impossible to tell from this chromogenic stain whether the CathB shown is found in microglia or neurons.
We thank the referee for this comment. We now include double stain images of the NPA patient for iba1 and CathB that confirm the identity of the CathB positive cells as microglia (new Supplementary Figure S8).

13. Description of osmotic mini-pump on page 11: it appears from the sentence that the Ca074Me was released s.c., shouldn't that be i.c.v?

The description of the osmotic mini-pump has been corrected to clarify that the pumps were implanted subcutaneously but were connected to the brain through a catheter to permit sustained Ca074Me release into the cerebrospinal fluid (highlighted text in pages 12 and 24).

2nd Editorial Decision	5th October 2018

2nd Editorial Decision   5th October 2018

Thank you for submitting a revised version of your manuscript. It has now been seen by two of the original referees and we have now received their comments, which are enclosed below for your information.

As you can see, they both find that all criticisms have been sufficiently addressed and recommend the manuscript for publication. However, before we can officially accept your manuscript there are a few editorial issues concerning text and figures that I would ask you to address in a final revised version.

REFEREE REPORTS

Referee #1:

The paper is in my opinion now ready for publication

Referee #3:

Major points
1. The question whether the absence of microglia influences the demyelination process has been convincingly addressed in Fig. S2.
2. The analysis of Cathepsin B levels and its proform in organotypic cerebellar slice cultures and

microglia of neonatal mice supports nicely the data obtained with BMDM. Significance was not reached for Cathepsin B in the slice culture and for Pro-Cathepsin B in the neonatal microglia supernatant, which could be due to the low number of samples analyzed or different ratios of autocatalytic processing depending on the model system. Thus, the text on p 12 "We found increased CathB levels in the cerebellar tissue and in the secreted media of ASMko compared to wt mice. CathB increase was prevented by the microglia depleting treatment with PLX." should be altered by differentiating between Pro-CathB and CathB.

Minor points
Our questions have been addressed satisfactory and we do not insist on a change of the title.

2nd Revision - authors' response   12th October 2018

Please find attached the final revised version of our manuscript entitled "Lipid induced lysosomal damage after demyelination corrupts microglia protective function in Lysosomal Storage Disorders" (EMBOJ-2018-99553) addressing the remaining editorial issues as follows:

Accepted   25th October 2018

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ⬇

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: María Dolores Ledesma & Enrique Gabandé Rodríguez
Journal Submitted to: EMBO JOURNAL
Manuscript Number: EMBOJ-2018-99553

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

➜ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
➜ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
➜ graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
➜ if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
➜ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

➜ a specification of the experimental system investigated (eg cell line, species name).
➜ the assay(s) and method(s) used to carry out the reported observations and measurements
➜ an explicit mention of the biological and chemical entity(ies) that are being measured.
➜ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

➜ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
➜ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
➜ a statement of how many times the experiment shown was independently replicated in the laboratory.
➜ definitions of statistical methods and measures:
 • common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
 • are tests one-sided or two-sided?
 • are there adjustments for multiple comparisons?
 • exact statistical test results, e.g., P values = x but not P values < x;
 • definition of 'center values' as median or average;
 • definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ⬇ (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size for the various experiments was determined according to typical numbers of observation in the respective fields (e.g. molecular and cellular biology, biochemistry, histology...) and our experience in terms of using how many pairs of mice or tissues to consistently observe the differences between genotypes.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For all experiments, the number of mice and independent experiments are indicated in the figures or corresponding legends.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No any animal or sample was excluded from the analysis
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Wild-type and mutant mice across all experiments were littermate pairs from heterozygous matings and housed together. In general, mice were assigned to experimental groups without special method.
For animal studies, include a statement about randomization even if no randomization was used.	All WT and ASMKO mice were littermates from heterozygous crosses and assigned parallel to experiments without specific criteria or randomization
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Mutants were always compared/processed in parallel to wild-type littermate controls to minimize subjective bias.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Blinding to genotypes was applied for behavioural tests performance and quantification
5. For every figure, are statistical tests justified as appropriate?	Yes, statistical analyses were conducted using GraphPad Prism or SPSS software with the recommended methods for testing normality and variances (e.g. Shapiro-Wilk test for normality) of data and post-hoc comparing (e.g. Bonferroni Dunnett's or Tukey's multiple comparison) statistical differences between groups. Details are explained in Matherials and methods

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http://www.selectagents.gov/

Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Data analyzed by ANOVA passed the normality test (Shapiro-Wilk test); single pair data was tested for normality and analyzed by Student's t-test or U test. Details are explained in Mathterials and Methods.
Is there an estimate of variation within each group of data?	All data are provided with standard error of the mean, as specified in the 'Materails and Mehotds' section and in figure legends.
Is the variance similar between the groups that are being statistically compared?	The variances between groups were similar.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All information about antibodies used in the study is Materials and Methods.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	BV2 cells were obtained from Dr. Francisco Muñoz (Universidad Pompeu Fabra). Cells grew and differentiated normally, so we did not test for mycoplasma contamination

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Materials and Methods: Animals
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Materials and Methods
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirm the compliance with the ARRIVE guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	No deposited data in this study.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No